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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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☐ *Additional inventors are being named on the ____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

Site-specific chemical modification of HIV fusion inhibitor peptides, and resultant conjugates

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* **24**

☒ Drawing(s) *Number of Sheets* **5**

☐ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number

☒ Other (specify) **sequence listing, acknowledgment postcard, certificate of mailing**

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Respectfully submitted,

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Date **03/15/2004**

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CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10) Applicant(s): **Bray and Zhang**			Docket No. **TRM-008**
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Invention: **Site-specific chemical modification of HIV fusion inhibitor peptides, and resultant conjugates**

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SITE-SPECIFIC CHEMICAL MODIFICATION OF HIV FUSION INHIBITOR PEPTIDES, AND RESULTANT CONJUGATES

FIELD OF INVENTION

The present invention relates to a method for the site-specific chemical modification of an HIV fusion inhibitor peptide such that one or more amine or amino groups of the peptide is chosen to be chemically protected, leaving one or more free amine or amino groups of the peptide chosen to be unprotected so as to be covalently coupled to an amine-reactive or amino-reactive polymer in forming a conjugate comprised of HIV fusion inhibitor peptide to which, in a specific site(s) of the peptide, is covalently coupled the polymer.

BACKGROUND OF THE INVENTION

It is now well known that cells can be infected by HIV through a process by which fusion occurs between the cellular membrane and the viral membrane. The generally accepted model of this process is that the viral envelope glycoprotein complex (gp120/gp41) interacts with cell surface receptors on the membranes of the target cells. Following binding of gp120 to cellular receptors (e.g., CD4 in combination with a chemokine co-receptor such as CCR-5 or CXCR-4), induced is a conformational change in the gp120/gp41 complex that allows gp41 to insert into the membrane of the target cell and mediate membrane fusion.

The amino acid sequence of gp41, and its variation among different strains of HIV, is well known. FIG.1 is a schematic representation of the generally accepted functional domains of gp41 (note the amino acid sequence numbers may vary slightly depending on the HIV strain). The fusion peptide (fusogenic domain) is believed to be involved in insertion into and disruption of the target cell membrane. The transmembrane domain, containing the transmembrane anchor sequence, is located at the C-terminal end of the protein. Between the fusion peptide and transmembrane anchor are two distinct regions, known as heptad repeat (HR) regions, each region having a plurality of heptads. The HR1 region, nearer to the N-terminal end of the protein than the HR2 region, has been generally described as comprising amino acid residues of SEQ ID NO:1, or polymorphisms thereof. The amino acid sequence comprising the HR1 region and the amino acid sequence comprising the HR2 region are each highly conserved regions in the HIV-1 envelope protein (Shu et al., 1999, *Biochemistry*, 38:5378-5385;

Hanna et al., 2002, *AIDS* 16:1603-8). The HR2 region has been generally described as comprising amino acid residues of SEQ ID NO:2, or polymorphisms thereof. As further shown in FIG.1, the HR regions have a plurality of 7 amino acid residue stretches or "heptads" (the 7 amino acids in each heptad designated "a" through "g"), with a predominance of hydrophobic residues at the first ("a") and fourth ("d") positions, charged residues frequently at the fifth ("e") and seventh ("g") positions, and with the amino acids in the "a" position and "d" position being primary determinants that influence the oligomeric state and strand orientation (see, e.g., Akey et al., 2001, *Biochemistry*, 40:6352-60).

It was discovered that peptides derived from the native sequence of either the HR1 region ("HR1 peptides") or HR2 region ("HR2 peptides") of HIV gp41 inhibit transmission of HIV to host cells both in *in vitro* assays and in *in vivo* clinical studies (see, e.g., Wild et al., 1994, *Proc. Natl. Acad. Sci. USA*, 91:9770-9774; U.S. Patent Nos. 5,464,933 and 5,656,480 licensed to the present assignee; and Kilby et al., 1998, *Nature Med.* 4:1302-1306. See also, e.g., U.S. Patent Nos. 6,258,782 and 6,348,568 assigned to the present assignee. The disclosures of these patents are herein incorporated by reference). More particularly, HR2 peptides, as exemplified by DP178 (also known as T20, enfuvirtide, and Fuzeon® ; SEQ ID NO:3), T651 (SEQ ID NO:4), T649 (SEQ ID NO:5), blocked infection of target cells with potencies of 0.5 ng/ml (EC50 against HIV-1_{LAI}; see, e.g., Lawless et al., 1996, *Biochemistry*, 35:13697-13708), 5 ng (IC50; HIV-1IIIB), and 2 ng (IC50; HIV-1IIIB), respectively. The respective amino acid sequences of T651 (SEQ ID NO:2) and T649 (SEQ ID NO:3) are also disclosed in U.S. Patent No. 6,479,055 (assigned to the present assignee; the disclosure of which is herein incorporated by reference).

Efforts have been made to improve the biological activity of HIV gp41-derived peptides, such as by trying to stabilize the helical structure of the peptide (see, e.g., Sia et al., 2002, *Proc. Natl. Acad. Sci. USA* 99:14664-14669; Otaka et al., 2002, *Angew. Chem. Int. Ed.* 41:2938-2940; U.S. Application Numbers 10/664021 & 60/534810 assigned to the present assignee, and the disclosures of these patents are herein incorporated by reference). Efforts have been also been made to improve the pharmacological properties of HIV fusion inhibitor peptides such as by PEGylation (see, e.g., U.S. Patent No. 5,464,933; U.S. Application Number 10/671282), and as a fusion protein (see, e.g. U.S. Patent No. 6,017,536 and PCT WO 03/066078).

Polymers have been used extensively to improve the pharmacokinetics and

pharmacodynamics (and hence, drug performance) of drugs such as peptides, proteins, and small molecules. The most widely used polymer for pharmaceutical applications is a polyol, poly(ethylene glycol) ("PEG"). PEGylation is the process by which the drug is chemically modified to result in the covalent attachment of one or more PEG molecules to the drug. The improved pharmacological and biological properties associated with PEGylation of drugs are well known in the pharmaceutical art. For example, PEGylation can increase therapeutic efficacy by means including, but not limited to, reducing degradation by proteolytic enzymes and thereby increasing drug concentration; increasing the size of the drug to which it is attached, thereby improving drug biodistribution; and shielding antigenic epitopes in reducing immunogenicity where desired. By increasing the therapeutic efficacy, reduced may be the frequency of dosing and/or the amount of drug need to achieve a therapeutic effect.

PEG, as a linear polyether, has a general structure of:

$HO\text{-}(CH_2\text{-}CH_2O)_n\text{-}CH_2CH_2\text{-}OH$ where n can typically range from about 10 to about 2000.

PEG, as a branched polyether, has a general structure of:

PEG-T-PEG
|
Z

wherein T is a linker or molecular bridge linking the PEG molecules, and Z is the functional group with chemically reactive moiety.

Many of the PEG modifications, in forming PEG derivatives (PEG and PEG derivatives are known in the art as "PEG"), are directed to the end groups ("functional groups") in adding or varying their chemically reactive functionalities to be used to covalently attach the PEG molecule to a drug. Various PEG derivatives are well known in the art (see, e.g., Li and Kao, *Biomacromolecules* 4:1055-1067, 2003; Roberts et al., *Advanced Drug Delivery Reviews* 54:459-476, 2002). To couple PEG to a drug, typically a functional group of the PEG molecule needs to be activated. The type of functional group is based upon the choice of reactive group on the drug to which the PEG molecule is to be coupled. Most commonly for proteins and peptides, the reactive group is present on an amino acid selected from the group consisting of an internal amino acid (e.g., having a side chain with a free reactive group; including, but not limited to, lysine, cysteine, glutamic acid, serine, threonine, and the like), the N-terminal amino acid (having a N-

terminal amine group as a free reactive group), a C-terminal amino acid (having a C-terminal carboxylic acid as a free reactive group), and a combination thereof. Of the sites of a peptide to be coupled to PEG, most frequently chosen is the N-terminal amine group ("alpha amine") of the peptide's N-terminal amino acid, and the epsilon amino group ("epsilon amine") of an internal lysine (a lysine found within the amino acid sequence which is not the N-terminal amino acid or the C-terminal amino acid of the peptide) or of lysine when the lysine is present at the N-terminus or C-terminus.

However, a problem arises with this frequent strategy for PEGylation. For example, lysine is one of the most prevalent amino acids in proteins. With respect to HIV fusion inhibitor peptide T20 (SEQ ID NO:1), for example, there are two internal lysine residues in this 36 amino acid residue peptide. Thus, with a plurality of lysine residues in the amino acid sequence (hence, a plurality of epsilon amines available to be reactive with activated PEG) and an alpha amine, there exists several sites to which the activated PEG with an amine reactive functional group can be covalently coupled to such peptide. The result of PEGylation of such a peptide is a heterogeneous mixture comprised of a population of several conjugates varying in the number of PEG molecules attached and in the sites of attachment. Heterogeneity of such a peptide-polymer conjugate is often an undesirable result. This is because the pharmacological and biological properties associated with PEGylation of peptides can be dependent on factors such as (a) the number of PEG molecules attached to the peptide, and (b) the location of the sites on the peptide to which PEG is coupled. For example, *in vitro* biological activity of PEGylated human growth hormone-releasing factor depended on both the site and degree of PEGylation. It is very difficult, if possible at all, to separate out the species of peptide-polymer conjugate (with the desired number of PEG molecules and desired site(s) of attachment) from a heterogeneous mixture using conventional separation techniques known in the art. Such separation attempts can add to the expense, time, and reagents needed for producing the peptide-poylmer conjugate. Multiple lysine residues in the amino acid sequence of a peptide to be PEGylated are perceived as such a problem that one method of site-specific PEGylation was developed which involved replacing the lysine residues with amino acids other than lysine which lack a side chain having a free amine (Onda et al., *Bioconjugate Chemistry* 14:480-487, 2003).

Thus, in the formation of conjugates comprised of an HIV fusion inhibitor peptide (containing one or more internal lysine residues in its amino acid sequence) and polymer, there is a need for a site-specific modification of the peptide so that a polymer

may be covalently coupled only to a specific site or specific sites on the peptide chosen by a person performing the conjugation. More specifically, in an HIV fusion inhibitor peptide containing more than one free amine group which is available for coupling to a polymer having amine-reactive functionality(s), it is desirable to chemically protect one or more selected amine groups, leaving the unprotected, free amine group(s) available for covalently coupling to polymer. Additionally, it would be advantageous to provide an HIV fusion inhibitor peptide which has PEG coupled in a site-specific manner to one or more desired sites in the peptide.

SUMMARY OF THE INVENTION

The present invention relates to a method for producing a substantially homogeneous conjugate comprised of HIV fusion inhibitor peptide and polymer, wherein one or more free amine groups of the synthetic peptide, containing multiple free amine groups, is blocked by a chemical protecting agent in the process of chemically synthesizing the synthetic peptide, allowing only the desired (predetermined) unblocked free amine group(s) of the synthetic peptide to be available for reaction with a polymer containing a amine-reactive functional group, in covalently coupling the synthetic peptide to the polymer in a site-specific chemical modification.

Another object of the present invention is to provide a method for site-specific PEGylation of an HIV fusion inhibitor peptide, wherein during the chemical synthesis of the synthetic peptide, certain free amine groups (e.g., one or more of an alpha amine or epsilon amine) which are desired to be protected against PEGylation are selectively blocked with a chemical protecting agent, thereby leaving only the free amine group(s) of the synthetic peptide, which are desired (predetermined) to be covalently coupled to PEG through chemical modification, available for PEGylation. It is a further object of the present invention to provide a substantially homogeneous composition comprising a PEGylated HIV fusion inhibitor peptide containing one or more amine groups blocked with a chemical protecting agent.

It is yet another object of the present invention to provide a substantially homogeneous composition comprising a PEGylated HIV fusion inhibitor peptide containing one or more amine groups blocked with a chemical protecting agent. It is a further object of the present invention to remove the chemical protecting agent (in a "deprotection" step) in providing a substantially homogeneous composition comprising a PEGylated HIV fusion inhibitor peptide which is PEGylated at only specific site(s), as

selected in performance of the method of the present invention, wherein such composition retains substantial anti-HIV activity. The present invention also provides for a method of treating HIV infection (preferably, HIV-1 infection) comprising administering to an HIV-infected individual a pharmaceutical composition comprising a substantially homogeneous conjugate comprised of an HIV fusion inhibitor peptide site-specifically coupled to a polymer. Preferably, the pharmaceutical composition is in an amount effective to inhibit transmission of HIV to a target cell, and/or in an amount effective to inhibit gp41-mediated fusion of HIV to a target cell. Also provided is a method for inhibition of transmission of HIV to a cell, comprising contacting the virus in the presence of a cell with the substantially homogeneous conjugate according to the present invention in an amount effective to inhibit infection of the cell by HIV. Additionally, provided is a method for inhibition of transmission of HIV to a cell, comprising adding to the virus and the cell an amount of the substantially homogeneous conjugate according to the present invention effective to inhibit infection of the cell by HIV. Also provided is a method for inhibiting HIV fusion (e.g., a process by which HIV gp41 mediates fusion between the viral membrane and cell membrane during infection by HIV of a target cell), comprising contacting the virus in the presence of a cell with an amount of the substantially homogeneous conjugate according to the present invention effective to inhibit HIV membrane fusion. These methods may be used to treat HIV-infected individuals.

It is a further object of the present invention to provide the use of the conjugates of the present invention in the manufacture of a medicament for use with the methods of inhibiting transmission of HIV, inhibiting HIV fusion, or treating HIV infection, as described herein. The medicament is preferably in the form of a pharmaceutical composition comprising a conjugate of the present invention together with a pharmaceutically acceptable carrier.

The above and other objects, features, and advantages of the present invention will be apparent in the following Detailed Description of the Invention when read in conjunction with accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic of HIV-1 gp41 showing the heptad repeat 1 region (HR1) and heptad repeat 2 region (HR2) along with other functional regions of gp41. Exemplary amino acid sequences corresponding to HR1 and HR2, and the amino acid position

numbering, are shown for purposes of illustration and in relation to gp160, strain HIV_{IIIB}.

FIG. 2 shows a comparison of the sequences contained within the HR1 region of HIV-1 gp41 for purposes of illustration, and not limitation, as determined from various laboratory strains and clinical isolates, wherein illustrated are some of the variations in amino acid sequence, as indicated by the single letter amino acid code.

FIG. 3 is a schematic showing synthesis of an HIV fusion inhibitor peptide using a fragment condensation approach.

FIG. 4 is a schematic showing synthesis of an HIV fusion inhibitor peptide using a fragment condensation approach, wherein the lysine residue at amino acid position 28 was chosen to be site-specifically conjugated to a polymer.

FIG. 5 is a schematic showing synthesis of an HIV fusion inhibitor peptide using a fragment condensation approach, wherein the lysine residue at amino acid position 18 was chosen to be site-specifically conjugated to a polymer.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "individual", when used herein for purposes of the specification and claims, means a mammal, and preferably a human.

The term "target cell", when used herein for purposes of the specification and claims, means a cell capable of being infected by HIV. Preferably, the cell is a human cell or are human cells; and more preferably, human cells capable of being infected by HIV via a process including membrane fusion.

The term "pharmaceutically acceptable carrier", when used herein for purposes of the specification and claims, means a carrier medium that does not significantly alter the biological activity of the active ingredient (e.g., drug or drug conjugate) to which it is added. A pharmaceutically acceptable carrier includes, but is not limited to, one or more of water, buffered water, saline, 0.3% glycine, aqueous alcohols, isotonic aqueous solution; and may further include one or more substances such as glycerol, oils, salts such as sodium, potassium, magnesium and ammonium, phosphonates, carbonate esters, fatty acids, saccharides (e.g., mannitol), polysaccharides, excipients, and preservatives and/or stabilizers (to increase shelf-life or as necessary and suitable for manufacture and distribution of the composition). Preferably, the pharmaceutically acceptable carrier is suitable for intravenous, intramuscular, subcutaneous or parenteral

administration.

By the term "amino acid" is meant, for purposes of the specification and claims and in reference to the synthetic peptides used in the present invention, to refer to a molecule that has at least one free amine group and at least one free carboxyl group. The amino acid may have more than one free amine group, or more than one free carboxyl group, or may further comprise one or more free chemical reactive groups other than an amine or a carboxyl group (e.g., a hydroxyl, a sulfhydryl, etc.). The amino acid may be a naturally occurring amino acid (e.g., L-amino acid), a non-naturally occurring amino acid (e.g., D-amino acid), a synthetic amino acid, a modified amino acid, an amino acid derivative, an amino acid precursor, and a conservative substitution. One skilled in the art would know that the choice of amino acids incorporated into a peptide will depend, in part, on the specific physical, chemical or biological characteristics required of the antiviral peptide. Such characteristics are determined, in part, by determination of structure and function (e.g., antiviral activity; as described herein in more detail). For example, the skilled artisan would know from the descriptions herein that amino acids in a synthetic peptide may be comprised of one or more of naturally occurring (L)-amino acid and non-naturally occurring (D)-amino acid. A preferred amino acid may be used to the exclusion of amino acids other than the preferred amino acid.

A "conservative substitution", in relation to amino acid sequence of a synthetic peptide used in the present invention, is a term used hereinafter for the purposes of the specification and claims to mean one or more amino acids substitution in the sequence of the synthetic peptide such that its biological activity is substantially unchanged (e.g., if the peptide inhibits HIV gp41-mediated fusion at a concentration in the nanomolar range before the substitution, after the substitution inhibition of HIV gp41-mediated fusion is still observed in the nanomolar range). As known in the art "conservative substitution" is defined by aforementioned function, and includes substitutions of amino acids having substantially the same charge, size, hydrophilicity, and/or aromaticity as the amino acid replaced. Such substitutions are known to those of ordinary skill in the art to include, but are not limited to, glycine-alanine-valine; isoleucine-leucine; tryptophan-tyrosine; aspartic acid-glutamic acid; arginine-lysine; asparagine-glutamine; and serine-threonine. With particular relevance to the present invention, a conserved substitution is known in the art to also include substituting lysine with ornithine, in providing a free amine group (e.g., epsilon amine). For HIV fusion inhibitor peptides, such substitutions may also comprise polymorphisms at the various amino acid positions along the relevant HR region (HR1 or

HR2) of gp41 found in laboratory and/or clinical isolates of HIV, which are readily available from public databases and are well known in the art (see also, FIG. 2, as an example).

The term "polymer" when used herein for purposes of the specification and claims, means a polymeric molecule which:(a) is employed in pharmaceutical applications to improve the pharmacological and/or biological properties when conjugated to a drug (and therefore is substantially nontoxic and substantially water soluble); (b) has one or more functional groups which by itself, and/or after activation to become chemically reactive, can be used to covalently couple to a free amine of the drug (e.g., a synthetic peptide) in forming a drug-polymer conjugate. Regarding the latter, the polymer preferably has an amine reactive group for covalently coupling to synthetic peptide. A polymer may include, but is not limited to, polylysines or poly(D-L-alanine)-poly(L-lysine)s, or polyols. A preferred polyol comprises a water-soluble poly(alkylene oxide) polymer, and can have a linear or branched chain. The term "polyol" is preferably a water-soluble, polyalcohol which may include, but is not limited to, poly-ethylene glycol ("PEG"), polypropylene glycol ("PPG"), diethylene glycol, triethylene glycol, ethylene glycol, dipropylene glycol, copolymers comprising PPG (e.g., ethylene glycol/PPG), copolymers comprising PEG (e.g., PEG/PPG), mPEG (monomethoxy-poly(ethylene) glycol), and the like. A polyol encompasses both homopolymers and copolymers, and further may have a structure comprising a branched structure or linear structure as known to those skilled in the art. Preferably, the polymer is substantially non-toxic when used for *in vivo* applications in individuals. In a preferred embodiment, the polymer has a molecular weight in the range between about 200 daltons to about 40,000 daltons; and in a more preferred embodiment, the polymer has a molecular weight range between about 400 daltons to about 10,000 daltons. A preferred polymer for application in the present invention comprises a polyethylene glycol ("PEG"), and a more preferred polymer for application in the present invention comprises a polyethylene glycol having a molecular weight range, wherein the molecular weight range is no less than about 400 daltons and is no more than about 20,000 daltons. As described previously herein, there are various forms of PEG that typically differ in the end groups or chemically reactive functional groups to be used to covalently attach the PEG molecule to a drug. Various PEGs are well known in the art (see, e.g., Li and Kao, *Biomacromolecules* 4:1055-1067, 2003; Roberts et al., *Advanced Drug Delivery Reviews* 54:459-476, 2002). A preferred PEG, for use in coupling to one

or more unprotected amine groups of the synthetic peptide in accordance with the present invention, has a chemically reactive group (e.g., functional group) which can be used covalently couple PEG to the to one or more unprotected amine groups. PEG may include but is not limited to, heterobifunctional PEG, PEG dichlorotriazine, PEG-tresylate, PEG succinimidyl carbonate, PEG benzotriazole carbonate, PEG *p*-nitrophenyl carbonate, PEG trichlorophenyl carbonate, PEG carbonylimidazole, PEG succinimidyl succinate, mPEG succinimidyl propionate, mPEG succinimidyl butanoate, PEG butyraldehyde, mPEG-propionaldehyde, PEG aldehyde, PEG-acetaldehyde, PEG acetaldehyde diethyl acetal, PEG carboxylic acid, mPEG phenyl ether succinimidyl carbonates, mPEG benzamide succinimidyl carbonates, PEG thioester, linear PEG, branched PEG, and linear forked PEG. A preferred polymer may be applied to the present invention to the exclusion of a polymer other than the preferred polymer.

The terms "synthetic peptide" and "HIV fusion inhibitor peptide" are used synonymously herein, in relation to a peptide employed in the present invention, and for the purposes of the specification and claims, to mean a peptide (a) comprising an amino acid sequence of no less than about 15 amino acids and no more than about 60 amino acid residues in length, and comprises at least 10 contiguous amino acids contained in either the HR1 region or HR2 region of gp41 of HIV (more preferably of HIV-1); and (b) capable of inhibiting transmission of HIV to a target cell (preferably, by complexing to an HR region of HIV-1 gp41 and inhibiting fusion between HIV-1 and a target cell), as can be determined by assessing antiviral activity *in vitro* and/or *in vivo*, as will be described in more detail herein. More preferably, the synthetic peptide employed in the present invention may comprise a sequence of no less than 28 amino acids and no more than about 51 amino acids in length, and even more preferably no less than about 36 amino acids and no more than about 51 amino acids in length. The term "isolated" when used in reference to a synthetic peptide means that it is substantially free of components which have not become part of the integral structure of the peptide itself; e.g., such as substantially free of chemical precursors or other chemicals when chemically synthesized or produced using biochemical or chemical processes. The synthetic peptide may comprise, in it's amino acid sequence, one or more conservative substitutions and/or one or polymorphisms found in the sequence of the relevant region of the HIV gp41, or may comprise one or more amino acid substitutions which are added to stabilize helix structure and/or affect oligomerization; provided that it retains antiviral activity against HIV-1 (e.g., an IC50 in the picpmolar to micromolar range). The

following are illustrative examples of HIV fusion inhibitor peptides that can be site-specifically conjugated to polymer in accordance with the present invention. However, a preferred synthetic peptide may be used in the present invention to the exclusion of a synthetic peptide other than the preferred synthetic peptide. As apparent to one skilled in the art and from the teachings herein, a lysine in the amino acid sequence of a synthetic peptide may be substituted with another amino acid having a side chain with a free amino group (e.g., epsilon amine). Ornithine is an illustrative example of such amino acid that may be used to substitute a lysine.

Preferably for use according to the present invention, for a synthetic peptide comprising sequence derived from the HR1 region of HIV-1 gp41, the synthetic peptide comprises a contiguous sequence of at least 15 amino acid residues in the amino acid sequence of SEQ ID NO:1, or polymorphisms thereof, as key determinants in this portion of the HR1 region (e.g., such as, noted by single letter amino acid designation, NNLLRAIEAQQHLLQLTVWG IKQLQARI LAVERYLKD which is amino acid residue 18 to amino acid residue 54 of SEQ ID NO:1) have been found to influence structure, and biochemical and antiviral parameters described herein. Note that there are two lysine residues internal to this portion of the HR1 region, one or more of which may be used for site-specifically coupling to a polymer according to the present invention. A preferred example of a synthetic peptide derived from the HR-1 region of HIV gp41, and as containing the amino acids found in the native sequence of this region, is illustrated as comprising an amino acid sequence of SEQ ID NO:6. Other examples of a synthetic peptide derived from the HR-1 region of HIV gp41, and as containing the amino acids found in the native sequence of this region, are illustrated as comprising amino acid sequences of SEQ ID NOs:7-22, and may further comprise an amino acid sequence having at least 90% identity with any one or more of SEQ ID NOs:6-22. More preferably for use according to the present invention, a synthetic peptide derived from the HR1 region of HIV gp41 contains one or more amino acid substitutions (e.g., as compared to the amino acid sequence of SEQ ID NO:1) which preferably enables the synthetic peptide to self-assemble into trimers (e.g., a trimer being comprised of three molecules of synthetic peptide), as disclosed in more detail in co-pending U.S. Application Number 10/664,021 (the disclosure of which is herein incorporated by reference). Examples of a synthetic peptide derived from the HR-1 region of HIV gp41 and which further comprises one or more amino acid substitutions which enable the synthetic peptide to self-assemble into trimers are illustrated as comprising amino acid sequences of SEQ ID

NOs:23-36, and may further comprise an amino acid sequence having at least 90% identity with any one or more of SEQ ID NOs: 23-36. Note that such synthetic peptides have one or more lysine residues internal to this portion of the HR1 region, one or more of which may be used for site-specifically coupling to a polymer according to the present invention.

Preferably for use according to the present invention, for a synthetic peptide comprising sequence derived from the HR2 region of HIV-1 gp41, the synthetic peptide comprises a contiguous sequence of at least amino acid residues 43 to 51of SEQ ID NO:2 (e.g., QQEKNEQEL), or polymorphisms thereof, as key determinants in this portion of the HR2 region have been found to influence biochemical and antiviral parameters described herein. Note there is one internal lysine residue in this sequence. Illustrative synthetic peptides derived from the HR2 region include, but are not limited to peptides having the amino acid sequences shown in SEQ ID NOs: 3, 4, 5, and 37 to 63, and may further comprise an amino acid sequence having at least 90% identity with any one or more of SEQ ID NOs:3, 4, 5, and 37 to 63. Note that such synthetic peptides have one or more internal lysine residues (and/or in the case of SEQ ID NOs. 34, 39 and 48, at the carboxy terminus), one or more of which may be used for site-specifically coupling to a polymer according to the present invention. More preferably for use according to the present invention, a synthetic peptide derived from the HR2 region of HIV gp41 contains one or more amino acid substitutions (e.g., as compared to a relative portion of the amino acid sequence of SEQ ID NO:2) which preferably promotes the helicity and/or helix stability the synthetic peptide in imparting improved biological activity, as disclosed in more detail in co-pending U.S. Application Number 60/534,810 (the disclosure of which is herein incorporated by reference). Examples of a synthetic peptide derived from the HR-2 region of HIV gp41 and which further comprises one or more amino acid substitutions for helix stability and improved biological activity are illustrated as comprising amino acid sequences of SEQ ID NOs:64-92, and may further comprise an amino acid sequence having at least 90% identity with any one or more of SEQ ID NOs: 64-92. Other examples of helix-stabilized peptides derived from the HR2 region of HIV gp41 may include SEQ ID NOs: 93-95. Note that such synthetic peptides have one or more internal lysine residues (and in some cases upwards to 25% of the amino acid sequence of the peptide), one or more of which may be used for site-specifically coupling to a polymer according to the present invention.

In another preferred embodiment according to the present invention, the

synthetic peptide may comprise a "hybrid" peptide comprising amino acid sequences derived from one or more of HIV-1, HIV-2, and SIV fusion proteins (see, e.g., U.S. Patent No. 6,258,782, the disclosure of which is herein incorporated by reference). Examples of a hybrid synthetic peptide are illustrated as comprising amino acid sequences of SEQ ID NOs:96 to112, and may further comprise an amino acid sequence having at least 90% identity with any one or more of SEQ ID NOs:96 to112. Note that such illustrated examples of hybrid synthetic peptides have at least two internal lysine residues, one or more of which may be used for site-specifically coupling to a polymer according to the present invention.

The term "percent identity", when used herein for purposes of the specification and claims in reference to a sequence according to the present invention, means that the sequence is compared ("Compared Sequence") to a described or reference sequence ("Reference Sequence"); wherein a percent identity is determined according to the following formula:

percent identity= [1-(xC/yR)] x 100

wherein xC is the number of differences between the Reference Sequence and the Compared Sequence over the length of alignment between the Compared Sequence and Reference Sequence wherein (a) each base or amino acid in the Reference Sequence that does not have a corresponding aligned base or amino acid compared to the Compared Sequence, and (b) each gap in the Reference Sequence, and (c) each aligned base or amino acid in the Compared Sequence that is different from an aligned base or amino acid in the Reference Sequence, constitutes a difference; and yR is the number of bases or amino acids in the Reference Sequence over the length of the Compared Sequence with any gap created in the Reference Sequence as a result of alignment also being counted as a base or amino acid. Methods and software for alignment between two predetermined sequences are well know in the art.

The term "chemical protecting agent", when used herein for purposes of the specification and claims, means a chemical moiety that is reactive with and can operably bind to a free amine of an amino acid, thereby blocking the amine from reacting with a polymer having a functional group that is amine-reactive. Preferably, the operably binding of the chemical moiety to the free amine group of the amino acid is reversible and, therefore, the chemical moiety may be removed from the amino acid in a process known in the art as "deprotection". More preferably, the chemical protecting agent can withstand removal of Fmoc (9-fluorenylmethoxycarbonyl) or Boc (tert-butyloxycarbonyl)

in a separate deprotection step, in remaining bound to the amine group until it is desired to remove the chemical protecting agent. A chemical protecting agent is known in the art to include, but is not limited to, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl ("Dde"), 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde), allyloxycarbonyl ("Alloc"), benzyloxycarbonyl ("Cbz"), and 2-Chlorobenzyloxycarbonyl ("2-Cl-Z").

The term "substantially homogeneous", when used herein for purposes of the specification and claims and in reference to a conjugate comprised of an HIV fusion inhibitor peptide coupled to a polymer produced according to the present invention, means that at least 95% of the resultant conjugate produced contains the HIV fusion inhibitor peptide site-specifically coupled to the polymer as intended by the orthogonal protection strategy employed, according to the method of the present invention. The conjugate may be further purified using separation technology including, but not limited to, chromatographic techniques known in the art.

The present invention provides a method for site-specific coupling of a polymer to an HIV fusion inhibitor peptide. The coupling of the polymer to the HIV fusion inhibitor peptide is via one or more free amine groups of the synthetic peptide available for chemical reaction with a polymer having a functional group which is amine reactive. For purposes of illustration, and not limitation, the following schematics demonstrates the method according to the present invention, and conjugates according to the present invention by using an exemplary HIV fusion inhibitor peptide known as T20 (SEQ ID NO:3).

Schematic 1: The synthetic peptide, being unmodified at the N-terminus after synthesis, has 3 free amine groups available for coupling to a polymer having an amine reactive functional group: the N-terminal amino acid alpha amine, and two internal lysine residues (labeled K_1 and K_2 for ease of description), each with a side chain having an epsilon amine.

Synthetic peptide: $^{2HN-}$YTSLIHSLIEESQNQQE K_1NEQELLELD K_2WASLWNWF

K_1: $\overset{|}{NH2}$ K_2: $\overset{|}{NH2}$

Schematic 2: The synthetic peptide shown in schematic 1 is conjugated to a polymer

("X") having an amine reactive functional group for coupling to the synthetic peptide. A heterogenous population of conjugates is possible from the conjugation process, as follows.

Conjugates: X-YTSLIHSLIEESQNQQE K_1NEQELLELD K_2WASLWNWF

^{2HN}-YTSLIHSLIEESQNQQE K_1NEQELLELD K_2WASLWNWF

X-YTSLIHSLIEESQNQQE K_1NEQELLELD K_2WASLWNWF

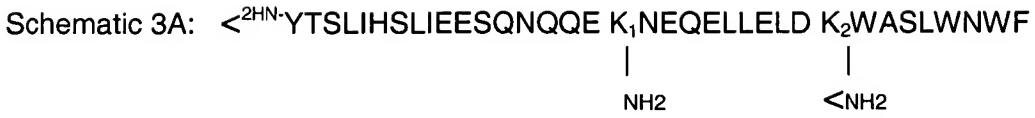
X-YTSLIHSLIEESQNQQE K_1NEQELLELD K_2WASLWNWF

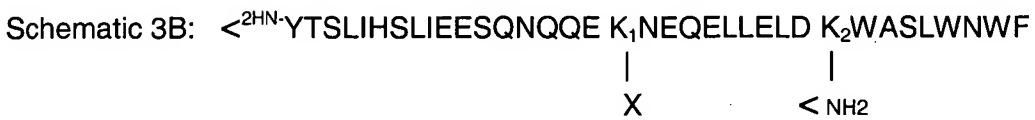
^{2HN}-YTSLIHSLIEESQNQQE K_1NEQELLELD K_2WASLWNWF

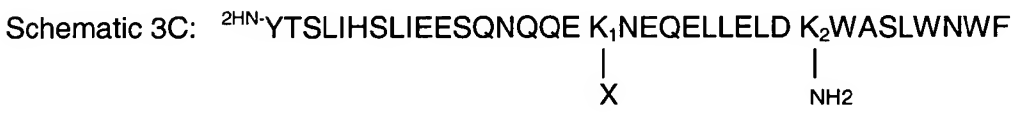
^{2HN}-YTSLIHSLIEESQNQQE K_1NEQELLELD K_2WASLWNWF

X-YTSLIHSLIEESQNQQE K_1NEQELLELD K_2WASLWNWF

Schematic 3: For illustration purposes, only the free amine of internal lysine residue "K_1" is selected (predetermined) to be coupled with a polymer having a amine reactive functional group in a method for site-specific chemical modification according to the present invention. First, the synthetic peptide is produced having the free amines desired to be blocked from reactivity with the polymer protected by a chemical protecting agent ("<") (schematic 3A); polymer is conjugated (schematic 3B); and the substantially homogenous conjugate is deprotected by removing the chemical protecting agent (schematic 3C).

Schematic 3A: 

Schematic 3B: 

Schematic 3C: 

The following Examples illustrate the present invention, and should not be construed as limiting the present invention.

EXAMPLE 1

Synthetic peptides may be typically be synthesized by linear synthesis on a peptide synthesizer using standard solid-phase synthesis techniques and using standard FMOC peptide chemistry or other standard peptide chemistry. However, in a preferred embodiment, T20 (SEQ ID NO:3) was synthesized by fragment condensation approach as previously described in more detail (see, e.g., U.S. Patent No. 6,015,881, the disclosure of which is herein incorporated by reference). Briefly, and as summarized in FIG. 3, a fragment of SEQ ID NO:3 comprising the first 16 amino acids was synthesized by standard solid phase synthesis (using a super acid sensitive resin); a fragment of SEQ ID NO:3 comprising amino acids 17-26 was synthesized by standard solid phase synthesis; and a fragment of SEQ ID NO:3 comprising amino acids 27-35 was synthesized by standard solid phase synthesis, followed by coupling to amino acid 36 in solution phase to result in a fragment comprising amino acids 27-36. The fragment of amino acids 17-26 was combined with the fragment of amino acids 27-36. The resulting amino acid sequence of amino acids 17-36 was combined with the fragment comprising amino acids 1-16 in forming a synthetic peptide comprising SEQ ID NO:3. The synthetic peptide was deprotected/decarboxylated, and then purified using reverse-phase high performance liquid chromatography. Peptide identity was confirmed with electrospray mass spectrometry.

Illustrated is one embodiment of a method for producing a substantially homogeneous conjugate comprised of HIV fusion inhibitor peptide and polymer, wherein

one or more free amine groups of the synthetic peptide, containing multiple free amine groups, is blocked by a chemical protecting agent in the process of chemically synthesizing the synthetic peptide, allowing only the desired unblocked free amine group(s) of the synthetic peptide to be available for reaction with a polymer containing a polymer in a site-specific chemical modification. In this illustrative embodiment, T20 (SEQ ID NO:3) was selected as the exemplary HIV fusion inhibitor peptide, and the lysine residue at amino acid position 28 ("Lys_{28}") was chosen to be site-specifically conjugated to a polymer. In referring to FIG.4, T20 (SEQ ID NO:3) was synthesized using the fragment condensation approach. Briefly, and as summarized in FIG. 4, a fragment of SEQ ID NO:3 comprising the first 16 amino acids was synthesized by standard solid phase synthesis with the N-terminal amine of amino acid residue 1 (Tyr) being acetylated ("Ac"). A fragment of SEQ ID NO:3 comprising amino acids 17-26 was synthesized by standard solid phase synthesis using Fmoc-Lys-(ivDde) as amino acid residue 18 ("Lys_{18}") , so that the chemical protecting agent ivDde blocks the epsilon amine group of Lys_{18} from reacting subsequently with a polymer having an amine reactive functional group. A fragment of SEQ ID NO:3 comprising amino acids 27-35 was synthesized by standard solid phase synthesis, and coupled to amnio acid 36 in solution phase in forming a fragment of amino acids 27-36. The fragment of amino acids 17-26 (with the ivDde protected Lys_{18}) was combined with the fragment of amino acids 27-36 (containing Lys_{28} with a free epsilon amine). The resulting amino acid sequence of amino acids 17-36 was combined with the fragment comprising amino acids 1-16 in forming a synthetic peptide comprising SEQ ID NO:3. The synthetic peptide SEQ ID NO:3 was deprotected with respect to removing protecting groups used in standard solid phase synthesis other than the chemical protecting agent ivDde, decarboxylated, and then purified using reverse-phase high performance liquid chromatography. To couple a polymer site-specifically to the free epsilon amine of Lys_{28} of SEQ ID NO:3, mPEG succinimidyl propionate ("mPEG –SPA") was chosen as the exemplary polymer. T20 (SEQ ID NO:3) with ivDde on epsilon amine group of Lys_{18} (9.0 mg, 2.0μmol) was dissolved in dimethyl formamide (DMF)(0.3 ml). Diisopropylethylamine (DIEA) (10μl) was added to the reaction, and then added was mPEG-SPA (average molecular weight, 5000 daltons; 20 mg, 4.0μmol) in DMF (1ml). The mixture was stirred at room temperature and the reaction is monitored by HPLC until the pegylation was completed. To remove the chemical protecting agent ivDde from the epsilon amine group of Lys_{18},

hydrazine (40μl) was added to the reaction to reach 3% (v/v) of hydrazine in reaction mixture. The stirring continued for another 30 minutes (or until HPLC shows deprotection is completed). The reaction mixture was diluted by water (6.5ml) to make the final concentration of DMF at 20%, then filtered through syringe filter (0.45μm, 2ml). HPLC purification was carried out on polystyrene/divinylbenzene column (PRLP-S, 300A, 10μm, 250*21.2mm) with acetonitrile-water-0.1% trifluoroacidic acid buffer as eluent. The collected fractions were checked by HPLC with both UV and ELS detectors. The pure fractions were pooled together and lyophilized for two days. The desired conjugate, a substantially homogeneous conjugate comprised of 5K-PEG-T20 at Lys_{28} was obtained as fluffy white solid (5.5mg) after lyophilization.

In another variation of this embodiment, the alpha amine of the N-terminal amino acid of SEQ ID NO:1 was not acetylated, but instead was protected with an Fmoc (9-fluorenylmethyloxycarbonyl) group. The process of synthetic peptide synthesis and conjugation to polymer was performed as provided in this Example 1. Thus, upon final deprotection, the conjugate comprised a substantially homogeneous conjugate comprised of 5K-PEG-T20 at Lys_{28}, except that the T20 (SEQ ID NO:3) of the conjugate contained a free alpha amine at the N-terminal amino acid (Tyr).

EXAMPLE 2

Illustrated is another embodiment of a method for producing a substantially homogeneous conjugate comprised of HIV fusion inhibitor peptide and polymer, wherein one or more free amine groups of the synthetic peptide, containing multiple free amine groups, is blocked by a chemical protecting agent in the process of chemically synthesizing the synthetic peptide, allowing only the desired unblocked free amine group(s) of the synthetic peptide to be available for reaction with a polymer containing a polymer in a site-specific chemical modification. In this illustrative embodiment, T20 (SEQ ID NO:3) was selected as the exemplary HIV fusion inhibitor peptide, and the lysine residue at amino acid position 18 ("Lys_{18}") was chosen to be site-specifically conjugated to a polymer. In referring to FIG.5, T20 (SEQ ID NO:3) was synthesized using the fragment condensation approach. Briefly, and as summarized in FIG. 5, a fragment of SEQ ID NO:3 comprising the first 16 amino acids was synthesized by standard solid phase synthesis with the N-terminal amine of amino acid residue 1 (Tyr) being acetylated ("Ac"). A fragment of SEQ ID NO:3 comprising amino acids 17-26 was

synthesized by standard solid phase synthesis. A fragment of SEQ ID NO:3 comprising amino acids 27-35 was synthesized by standard solid phase synthesis using Fmoc-Lys-(ivDde) as amino acid residue 28 ("Lys_{28}") , so that the chemical protecting agent ivDde blocks the epsilon amine group of Lys_{28} from reacting subsequently with a polymer having an amine reactive functional group. The latter fragment was coupled to amino acid 36 in solution phase to form a fragment containing amino acids 27-36. The fragment of amino acids 17-26 (containing Lys_{18} with a free epsilon amine) was combined with the fragment of amino acids 27-36 (with the ivDde protected Lys_{28}). The resulting amino acid sequence of amino acids 17-36 was combined with the fragment comprising amino acids 1-16 in forming a synthetic peptide comprising SEQ ID NO:3. The synthetic peptide SEQ ID NO:3 was deprotected with respect to removing protecting groups used in standard solid phase synthesis other than the chemical protecting agent ivDde, decarboxylated, and then purified using reverse-phase high performance liquid chromatography. To couple a polymer site-specifically to the free epsilon amine of Lys_{18} of SEQ ID NO:3, mPEG succinimidyl propionate ("mPEG –SPA") was chosen as the exemplary polymer. T20 (SEQ ID NO:3) with ivDde on epsilon amine group of Lys_{28} (19.7 mg, 4.4μmol) was dissolved in DMF (0.5 ml). DIEA (20μl) was added to the reaction, and then added was mPEG-SPA (average molecular weight, 5000 daltons; 50 mg, 10μmol) in DMF (1ml). The mixture was stirred at room temperature and the reaction is monitored by HPLC until the pegylation was completed. To remove the chemical protecting agent ivDde from the epsilon amine group of Lys_{28}, hydrazine (45μl) was added to the reaction to reach 3% (v/v) of hydrazine in reaction mixture. The stirring continued for another 30 minutes (or until HPLC shows deprotection is completed). The reaction mixture was diluted by water (6.5ml) to make the final concentration of DMF at 20%, then filtered through syringe filter (0.45μm, 2ml). HPLC purification was carried out on polystyrene/divinylbenzene column (PRLP-S, 300A, 10μm, 250*21.2mm) with acetonitrile-water-0.1% trifluoroacidic acid buffer as eluent. The collected fractions were checked by HPLC with both UV and ELS detectors. The pure fractions were pooled together and lyophilized for two days. The desired conjugate, a substantially homogeneous conjugate comprised of 5K-PEG-T20 at Lys_{18} was obtained as fluffy white solid (10.4mg) after lyophilization.

In another variation of this embodiment, the alpha amine of the N-terminal amino acid of SEQ ID NO:1 was not acetylated, but instead was protected with an Fmoc (9-

fluorenylmethyloxycarbonyl) group. The process of synthetic peptide synthesis and conjugation to polymer was performed as provided in this Example 2. Thus, upon final deprotection, the conjugate comprised a substantially homogeneous conjugate comprised of 5K-PEG-T20 at Lys_{18}, except that the T20 (SEQ ID NO:3) of the conjugate contained a free alpha amine at the N-terminal amino acid (Tyr).

EXAMPLE 3

Illustrated in this example is a method for determining the antiviral activity using the conjugates according to the present invention. In using an *in vitro* assay for demonstrating antiviral potency, it is important to note that antiviral effect of synthetic peptide demonstrated in the *in vitro* assay has been correlated with the antiviral effect of the synthetic peptide *in vivo*. In determining antiviral activity (e.g., one measure being the ability to inhibit transmission of HIV to a target cell) of the conjugates according to the present invention, used is an *in vitro* assay which has been shown, by data generated using synthetic peptides derived from either of the HR regions of HIV gp41, to be predictive of antiviral activity observed *in vivo*. More particularly, antiviral activity observed using an *in vitro* infectivity assay ("Magi-CCR5 infectivity assay"; see, e.g., U.S. Patent No. 6,258,782) has been shown to reasonably correlate to antiviral activity observed *in vivo* for the same HIV fusion inhibitor peptides (see, e.g., Kilby et al., 1998, *Nature Med.* 4:1302-1307). To further emphasize this point, T20 (SEQ ID NO:3) and T1249 (SEQ. ID NO:96) each have demonstrated potent antiviral activity against HIV in both the *in vitro* infectivity assay and human clinical trials.

The infectivity assays score for reduction of infectious virus titer employing the indicator cell lines MAGI or the CCR5 expressing derivative cMAGI. Both cell lines exploit the ability of HIV-1 tat to transactivate the expression of a β-galactosidase reporter gene driven by the HIV-LTR. The β-gal reporter has been modified to localize in the nucleus and can be detected with the X-gal substrate as intense nuclear staining within a few days of infection. The number of stained nuclei can thus be interpreted as equal to the number of infectious virions in the challenge inoculum if there is only one round of infection prior to staining. Infected cells are enumerated using a CCD-imager and both primary and laboratory adapted isolates show a linear relationship between virus input and the number of infected cells visualized by the imager. In the MAGI and cMAGI assays, a 50% reduction in infectious titer (Vn/Vo = 0.5) is significant, and provides the primary cutoff value for assessing antiviral activity ("IC50" is defined as the

dilution resulting in a 50% reduction in infectious virus titer). A secondary cutoff of Vn/Vo = 0.1, corresponding to a 90% reduction in infectious titer is also assessed ("IC90"). Conjugates tested for antiviral activity are diluted into various concentrations, and tested in duplicate or triplicate against an HIV inoculum adjusted to yield approximately 1500-2000 infected cells/well of a 48 well microtiter plate. The conjugate (in the respective dilution) is added to the cMAGI or MAGI cells, followed by the virus inocula; and 24 hours later, an inhibitor of infection and cell-cell fusion (e.g., T20) is added to prevent secondary rounds of HIV infection and cell-cell virus spread. The cells are cultured for 2 more days, and then fixed and stained with the X-gal substrate to detect HIV-infected cells. The number of infected cells for each control and conjugate dilution was determined with the CCD-imager, and then the IC50 and IC90 is calculated (typically expressed in μg/ml).

EXAMPLE 4

The present invention provides for conjugates comprised of an HIV fusion inhibitor peptide to which is coupled site-specifically a polymer. Antiviral activity of such conjugates can be utilized in a method for inhibiting transmission of HIV to a target cell, comprising adding to the virus and cell an amount of conjugate according to the present invention effective to inhibit infection of the cell by HIV, and more preferably, to inhibit fusion between the virus and the target cell. This method may be used to treat HIV-infected individuals (therapeutically) or to treat individuals newly exposed to or at high risk of exposure (e.g., through drug usage or high risk sexual behavior) to HIV (prophylactically). Thus, for example, in the case of an HIV-1 infected individual, an effective amount of conjugate would be a dose sufficient (by itself and/or in conjunction with a regimen of doses) to reduce HIV viral load in the individual being treated. As known to those skilled in the art, there are several standard methods for measuring HIV viral load which include, but are not limited to, by quantitative cultures of peripheral blood mononuclear cells and by plasma HIV RNA measurements. The conjugates of the invention can be administered in a single administration, intermittently, periodically, or continuously, as can be determined by a medical practitioner, such as by monitoring viral load. Depending on the formulation containing conjugate, and such factors as the compositions of the polymer and synthetic peptide used in forming the conjugate and whether or not further comprising a pharmaceutically acceptable carrier and the nature of the pharmaceutically acceptable carrier, the conjugate according to the present

invention may be administered with a periodicity ranging from days to weeks or possibly longer. Further, a conjugate according to the present invention may show synergistic results, of inhibiting transmission of HIV to a target cell, when used in combination (e.g., when used simultaneously, or in a cycling on with one drug and cycling off with another) with other antiviral drugs used for treatment of HIV (e.g., including, but not limited to, other HIV entry inhibitors (e.g., CCR5 inhibitors, retrocyclin, and the like), HIV integrase inhibitors, reverse transcriptase inhibitors (e.g., nucleoside or nonnucleoside), protease inhibitors, and the like, as well known in the art).

Effective dosages of a conjugate of the invention to be administered may be determined through procedures well known to those in the art; e.g., by determining potency, biological half-life, bioavailability, and toxicity. In a preferred embodiment, an effective conjugate dosage range is determined by one skilled in the art using data from routine *in vitro* and *in vivo* studies well know to those skilled in the art. For example, *in vitro* infectivity assays of antiviral activity, such as described herein, enables one skilled in the art to determine the mean inhibitory concentration (IC) of the conjugate necessary to block some amount of viral infectivity (e.g., 50% inhibition, IC_{50}; or 90% inhibition, IC_{90}). Appropriate doses can then be selected by one skilled in the art using pharmacokinetic data from one or more standard animal models, so that a minimum plasma concentration (C[min]) of the conjugate is obtained which is equal to or exceeds a predetermined IC value. While dosage ranges typically depend on the route of administration chosen and the formulation of the dosage, an exemplary dosage range of the conjugate according to the present invention may range from no less than 0.1 μg/kg body weight and no more than 10 mg/kg body weight; preferably a dosage range of from about 0.1-100 μg/kg body weight; and more preferably, a dosage of between from about 10 mg to about 250 mg of conjugate.

A conjugate of the present invention may be administered to an individual by any means that enables the active agent to reach the target cells (cells that can be infected by HIV). Thus, the conjugates of this invention may be administered by any suitable technique, including oral, parenteral (e.g., intramuscular, intraperitoneal, intravenous, or subcutaneous injection or infusion, intradermal, or implant), nasal, pulmonary, vaginal, rectal, sublingual, or topical routes of administration, and can be formulated in dosage forms appropriate for each route of administration. The specific route of administration will depend, e.g., on the medical history of the individual, including any perceived or anticipated side effects from such administration, and the formulation of conjugate being

administered (e.g., the nature of the polymer and synthetic peptide of which the conjugate comprises). Most preferably, the administration is by injection (using, e.g., intravenous or subcutaneous means), but could also be by continuous infusion (using, e.g., slow-release devices or minipumps such as osmotic pumps, and the like). A conjugate according to the present invention may further comprise a pharmaceutically acceptable carrier; and may further depend on the formulation desired, site of delivery, the method of administration, the scheduling of administration, and other factors known to medical practitioners.

The foregoing description of the specific embodiments of the present invention have been described in detail for purposes of illustration. In view of the descriptions and illustrations, others skilled in the art can, by applying current knowledge, readily modify and/or adapt the present invention for various applications without departing from the basic concept; and thus, such modifications and/or adaptations are intended to be within the meaning and scope of the appended claims.

What is claimed is:

1. A method for producing a substantially homogeneous conjugate comprised of HIV fusion inhibitor peptide and polymer, wherein the HIV fusion inhibitor peptide comprises more than one free amine group, and wherein the polymer is to be coupled to at least one predetermined site of the HIV fusion inhibitor peptide in site-specifically coupling the polymer to the HIV fusion inhibitor peptide, the method comprising:

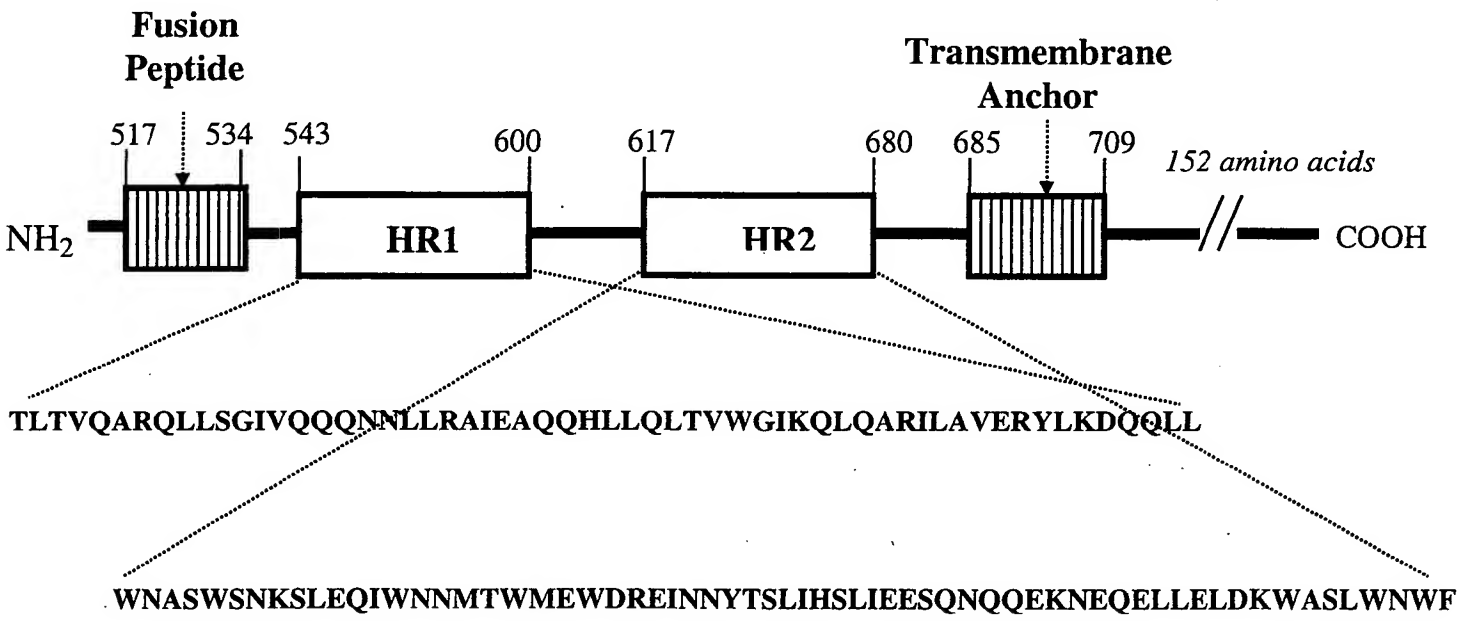
(a) synthesizing the HIV fusion inhibitor peptide so that one or more of the free amine groups, which does not comprise the at least one predetermined site, is blocked by a chemical protecting agent;

(b) chemically coupling a polymer having at least one amine reactive functional group to the one or more free amine groups of the HIV fusion inhibitor peptide, which comprise the at least one predetermined site and which is not blocked by the chemical protecting agent, in producing a substantially homogeneous conjugate comprised of HIV fusion inhibitor peptide and polymer.

ABSTRACT

Provided is a method for producing a substantially homogeneous conjugate comprised of HIV fusion inhibitor peptide and polymer, wherein the HIV fusion inhibitor peptide, comprising more than one free amine group, is synthesized so that one or more predetermined amine groups remain free to be reacted with a polymer having an amine reactive functional group, and the remaining one or more amine groups of the HIV fusion inhibitor peptide is blocked by a chemical protecting agent. The polymer is then chemically coupled to the one or more free (unprotected) amine groups of the HIV fusion inhibitor peptide in producing a substantially homogeneous conjugate comprised of HIV fusion inhibitor peptide and polymer.

FIG. 1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543
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617
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HR1
HR2
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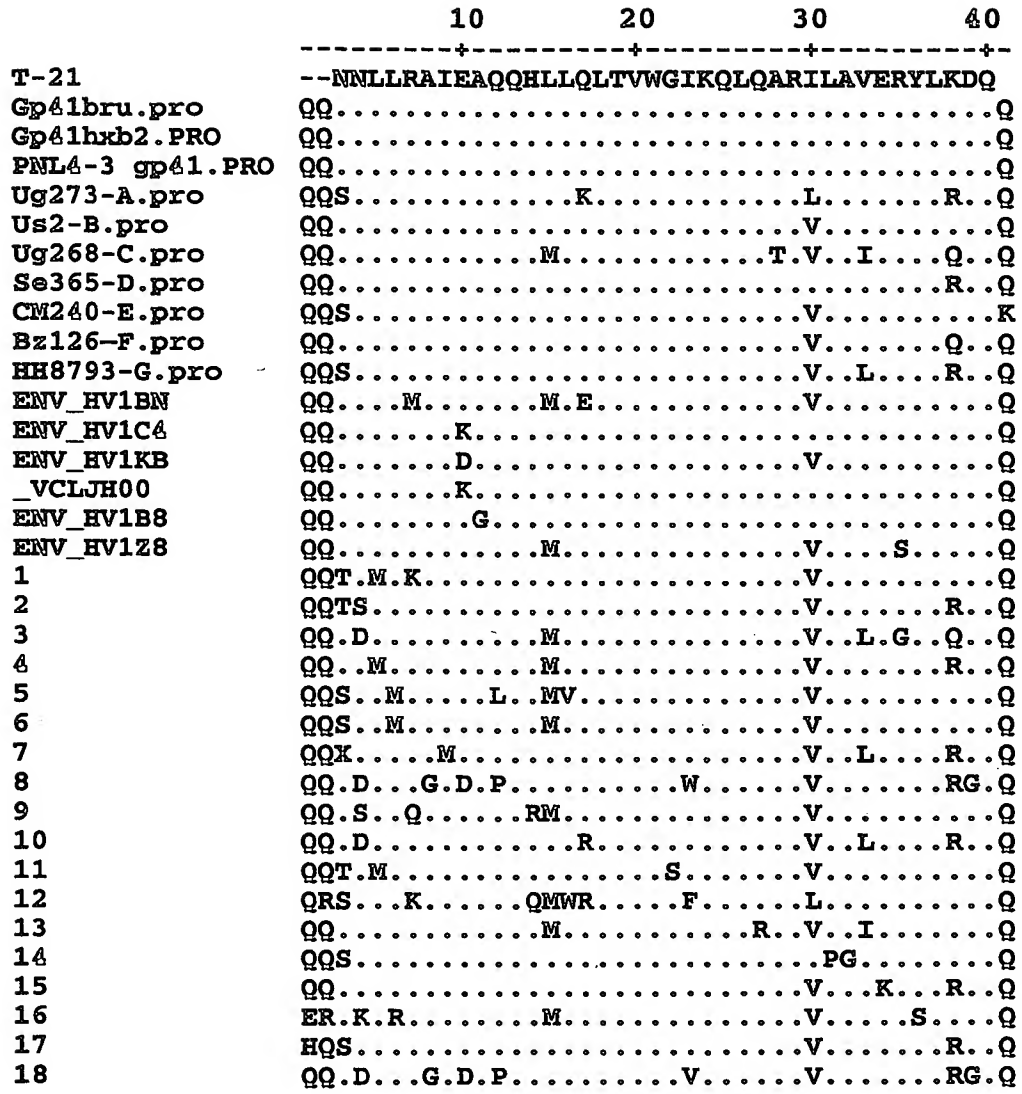
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Ug273-A.pro QQS.....K.....L.....R..Q
Us2-B.pro QQ.....V.....Q
Ug268-C.pro QQ.....M.....T.V..I....Q..Q
Se365-D.pro QQ.....R..Q
CM240-E.pro QQS.....V.....K
Bz126-F.pro QQ.....V.....Q..Q
HH8793-G.pro QQS.....V..L....R..Q
ENV_HV1BN QQ....M.....M.E.....V.....Q
ENV_HV1C4 QQ.....K.....Q
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_VCLJH00 QQ.....K.....Q
ENV_HV1B8 QQ.....G.....Q
ENV_HV1Z8 QQ.....M.....V....S.....Q
1 QQT.M.K.....V.....Q
2 QQTS.....V.....R..Q
3 QQ.D.....M.....V..L.G..Q..Q
4 QQ..M.....M.....V.....R..Q
5 QQS..M.....L..MV.....V.....Q
6 QQS..M.....M.....V.....Q
7 QQX.....M.....V..L....R..Q
8 QQ.D...G.D.P.....W.....V.....RG.Q
9 QQ.S..Q.....RM.....V.....Q
10 QQ.D.....R.....V..L....R..Q
11 QQT.M.....S.....V.....Q
12 QRS...K.....QMWR.....F.....L.....Q
13 QQ.....M.....R..V..I.....Q
14 QQS.....PG.....Q
15 QQ.....V...K...R..Q
16 ER.K.R.....M.....V.....S....Q
17 HQS.....V.....R..Q
18 QQ.D...G.D.P.....V.....V.....RG.Q

FIG. 2

FIG. 3

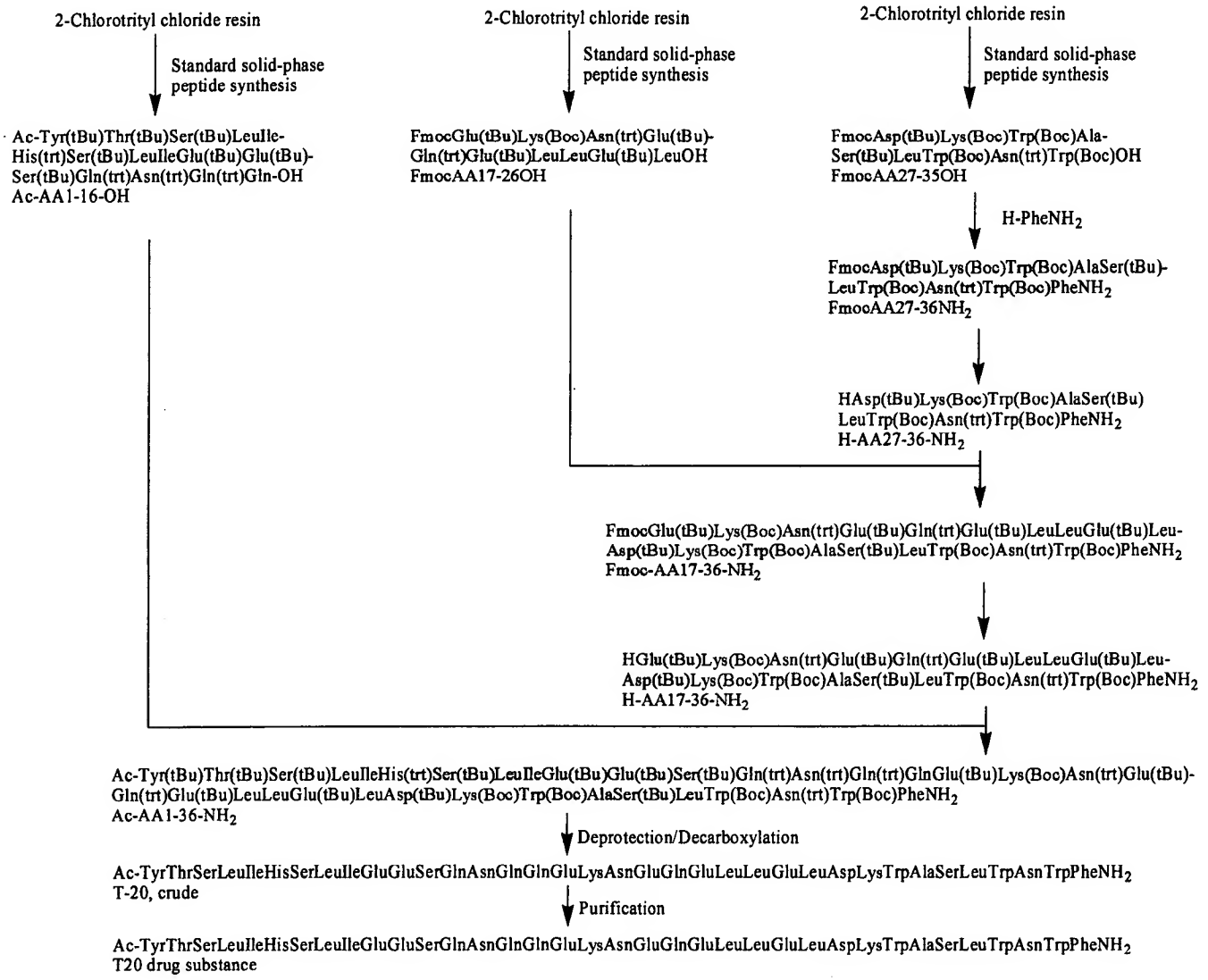

2-Chlorotrityl chloride resin
Standard solid-phase peptide synthesis
Ac-Tyr(tBu)Thr(tBu)Ser(tBu)LeuIle-His(trt)Ser(tBu)LeuIleGlu(tBu)Glu(tBu)-Ser(tBu)Gln(trt)Asn(trt)Gln(trt)Gln-OH
Ac-AA1-16-OH
2-Chlorotrityl chloride resin
Standard solid-phase peptide synthesis
FmocGlu(tBu)Lys(Boc)Asn(trt)Glu(tBu)-Gln(trt)Glu(tBu)LeuLeuGlu(tBu)LeuOH
FmocAA17-26OH
2-Chlorotrityl chloride resin
Standard solid-phase peptide synthesis
FmocAsp(tBu)Lys(Boc)Trp(Boc)Ala-Ser(tBu)LeuTrp(Boc)Asn(trt)Trp(Boc)OH
FmocAA27-35OH
H-PheNH2
FmocAsp(tBu)Lys(Boc)Trp(Boc)AlaSer(tBu)-LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
FmocAA27-36NH2
HAsp(tBu)Lys(Boc)Trp(Boc)AlaSer(tBu)
LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
H-AA27-36-NH2
FmocGlu(tBu)Lys(Boc)Asn(trt)Glu(tBu)Gln(trt)Glu(tBu)LeuLeuGlu(tBu)Leu-Asp(tBu)Lys(Boc)Trp(Boc)AlaSer(tBu)LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
Fmoc-AA17-36-NH2
HGlu(tBu)Lys(Boc)Asn(trt)Glu(tBu)Gln(trt)Glu(tBu)LeuLeuGlu(tBu)Leu-Asp(tBu)Lys(Boc)Trp(Boc)AlaSer(tBu)LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
H-AA17-36-NH2
Ac-Tyr(tBu)Thr(tBu)Ser(tBu)LeuIleHis(trt)Ser(tBu)LeuIleGlu(tBu)Glu(tBu)Ser(tBu)Gln(trt)Asn(trt)Gln(trt)GlnGlu(tBu)Lys(Boc)Asn(trt)Glu(tBu)-Gln(trt)Glu(tBu)LeuLeuGlu(tBu)LeuAsp(tBu)Lys(Boc)Trp(Boc)AlaSer(tBu)LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
Ac-AA1-36-NH2
Deprotection/Decarboxylation
Ac-TyrThrSerLeuIleHisSerLeuIleGluGluSerGlnAsnGlnGlnGluLysAsnGluGlnGluLeuLeuGluLeuAspLysTrpAlaSerLeuTrpAsnTrpPheNH2
T-20, crude
Purification
Ac-TyrThrSerLeuIleHisSerLeuIleGluGluSerGlnAsnGlnGlnGluLysAsnGluGlnGluLeuLeuGluLeuAspLysTrpAlaSerLeuTrpAsnTrpPheNH2
T20 drug substance

FIG. 4

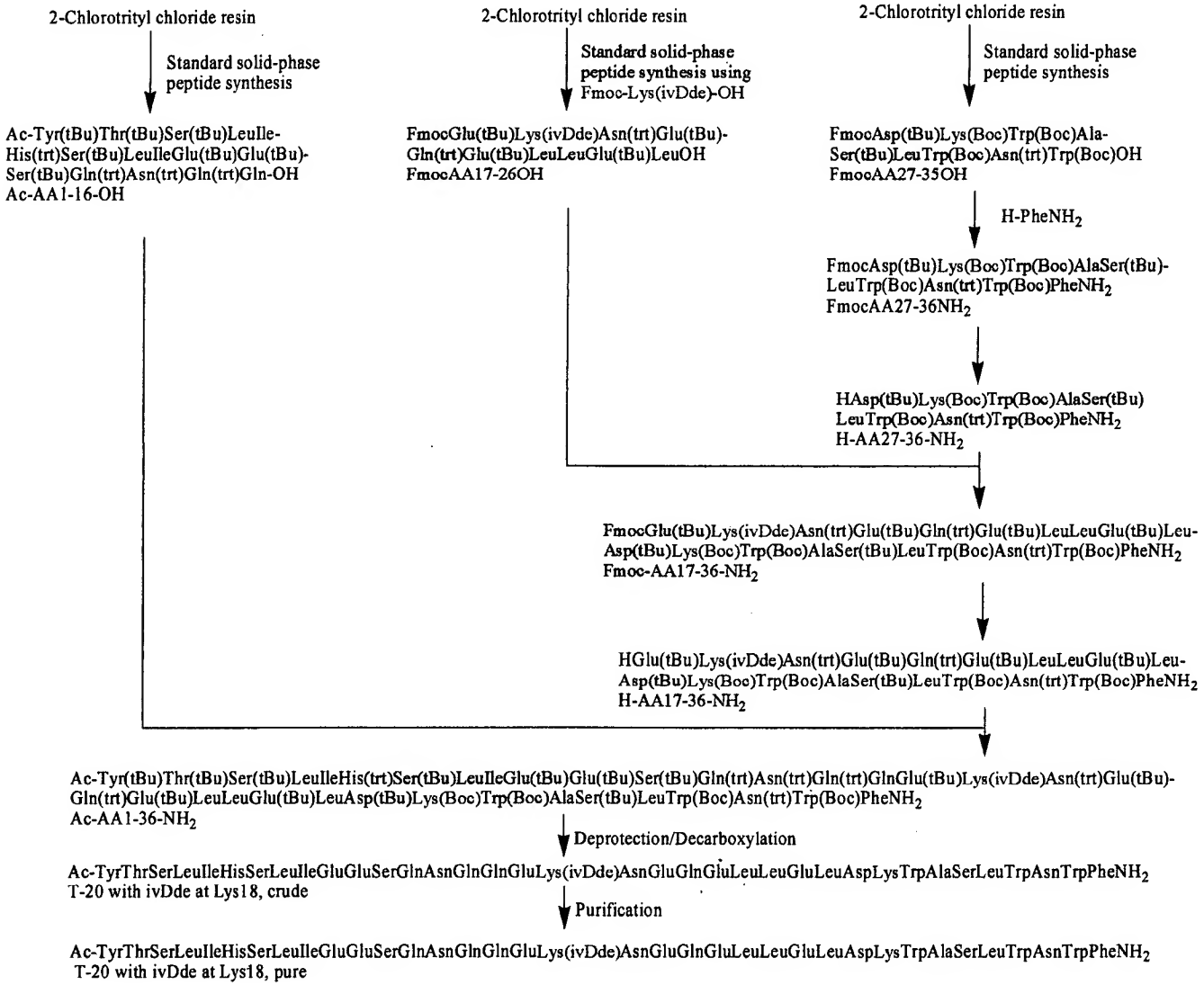
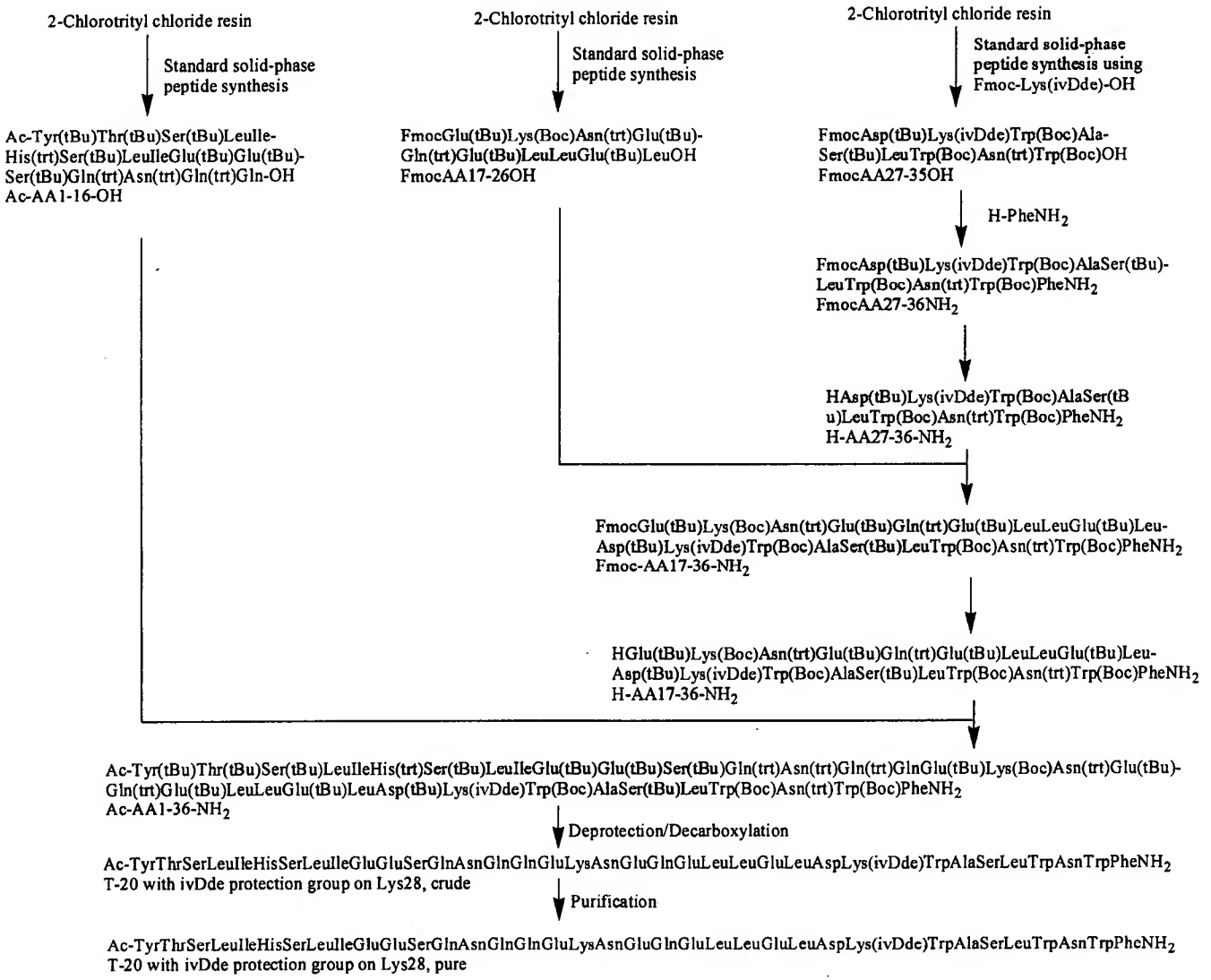

2-Chlorotrityl chloride resin
Standard solid-phase peptide synthesis
Ac-Tyr(tBu)Thr(tBu)Ser(tBu)LeuIle-His(trt)Ser(tBu)LeuIleGlu(tBu)Glu(tBu)-Ser(tBu)Gln(trt)Asn(trt)Gln(trt)Gln-OH
Ac-AA1-16-OH
2-Chlorotrityl chloride resin
Standard solid-phase peptide synthesis using Fmoc-Lys(ivDde)-OH
FmocGlu(tBu)Lys(ivDde)Asn(trt)Glu(tBu)-Gln(trt)Glu(tBu)LeuLeuGlu(tBu)LeuOH
FmocAA17-26OH
2-Chlorotrityl chloride resin
Standard solid-phase peptide synthesis
FmocAsp(tBu)Lys(Boc)Trp(Boc)Ala-Ser(tBu)LeuTrp(Boc)Asn(trt)Trp(Boc)OH
FmocAA27-35OH
H-PheNH2
FmocAsp(tBu)Lys(Boc)Trp(Boc)AlaSer(tBu)-LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
FmocAA27-36NH2
HAsp(tBu)Lys(Boc)Trp(Boc)AlaSer(tBu)
LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
H-AA27-36-NH2
FmocGlu(tBu)Lys(ivDde)Asn(trt)Glu(tBu)Gln(trt)Glu(tBu)LeuLeuGlu(tBu)Leu-Asp(tBu)Lys(Boc)Trp(Boc)AlaSer(tBu)LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
Fmoc-AA17-36-NH2
HGlu(tBu)Lys(ivDde)Asn(trt)Glu(tBu)Gln(trt)Glu(tBu)LeuLeuGlu(tBu)Leu-Asp(tBu)Lys(Boc)Trp(Boc)AlaSer(tBu)LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
H-AA17-36-NH2
Ac-Tyr(tBu)Thr(tBu)Ser(tBu)LeuIleHis(trt)Ser(tBu)LeuIleGlu(tBu)Glu(tBu)Ser(tBu)Gln(trt)Asn(trt)Gln(trt)GlnGlu(tBu)Lys(ivDde)Asn(trt)Glu(tBu)-Gln(trt)Glu(tBu)LeuLeuGlu(tBu)LeuAsp(tBu)Lys(Boc)Trp(Boc)AlaSer(tBu)LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
Ac-AA1-36-NH2
Deprotection/Decarboxylation
Ac-TyrThrSerLeuIleHisSerLeuIleGluGluSerGlnAsnGlnGlnGluLys(ivDde)AsnGluGlnGluLeuLeuGluLeuAspLysTrpAlaSerLeuTrpAsnTrpPheNH2
T-20 with ivDde at Lys18, crude
Purification
Ac-TyrThrSerLeuIleHisSerLeuIleGluGluSerGlnAsnGlnGlnGluLys(ivDde)AsnGluGlnGluLeuLeuGluLeuAspLysTrpAlaSerLeuTrpAsnTrpPheNH2
T-20 with ivDde at Lys18, pure

FIG. 5


2-Chlorotrityl chloride resin
Standard solid-phase peptide synthesis
Ac-Tyr(tBu)Thr(tBu)Ser(tBu)Leulle-His(trt)Ser(tBu)LeulleGlu(tBu)Glu(tBu)-Ser(tBu)Gln(trt)Asn(trt)Gln(trt)Gln-OH
Ac-AA1-16-OH
2-Chlorotrityl chloride resin
Standard solid-phase peptide synthesis
FmocGlu(tBu)Lys(Boc)Asn(trt)Glu(tBu)-Gln(trt)Glu(tBu)LeuLeuGlu(tBu)LeuOH
FmocAA17-26OH
2-Chlorotrityl chloride resin
Standard solid-phase peptide synthesis using Fmoc-Lys(ivDde)-OH
FmocAsp(tBu)Lys(ivDde)Trp(Boc)Ala-Ser(tBu)LeuTrp(Boc)Asn(trt)Trp(Boc)OH
FmocAA27-35OH
H-PheNH2
FmocAsp(tBu)Lys(ivDde)Trp(Boc)AlaSer(tBu)-LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
FmocAA27-36NH2
HAsp(tBu)Lys(ivDde)Trp(Boc)AlaSer(tBu)LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
H-AA27-36-NH2
FmocGlu(tBu)Lys(Boc)Asn(trt)Glu(tBu)Gln(trt)Glu(tBu)LeuLeuGlu(tBu)Leu-Asp(tBu)Lys(ivDde)Trp(Boc)AlaSer(tBu)LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
Fmoc-AA17-36-NH2
HGlu(tBu)Lys(Boc)Asn(trt)Glu(tBu)Gln(trt)Glu(tBu)LeuLeuGlu(tBu)Leu-Asp(tBu)Lys(ivDde)Trp(Boc)AlaSer(tBu)LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
H-AA17-36-NH2
Ac-Tyr(tBu)Thr(tBu)Ser(tBu)LeuIleHis(trt)Ser(tBu)LeuIleGlu(tBu)Glu(tBu)Ser(tBu)Gln(trt)Asn(trt)Gln(trt)GlnGlu(tBu)Lys(Boc)Asn(trt)Glu(tBu)-Gln(trt)Glu(tBu)LeuLeuGlu(tBu)LeuAsp(tBu)Lys(ivDde)Trp(Boc)AlaSer(tBu)LeuTrp(Boc)Asn(trt)Trp(Boc)PheNH2
Ac-AA1-36-NH2
Deprotection/Decarboxylation
Ac-TyrThrSerLeuIleHisSerLeuIleGluGluSerGlnAsnGlnGlnGluLysAsnGluGlnGluLeuLeuGluLeuAspLys(ivDde)TrpAlaSerLeuTrpAsnTrpPheNH2
T-20 with ivDde protection group on Lys28, crude
Purification
Ac-TyrThrSerLeuIleHisSerLeuIleGluGluSerGlnAsnGlnGlnGluLysAsnGluGlnGluLeuLeuGluLeuAspLys(ivDde)TrpAlaSerLeuTrpAsnTrpPheNH2
T-20 with ivDde protection group on Lys28, pure

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